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14. ABSTRACT

The purpose of this proposal is to develop an '*in vitro* cell-based system' to study radioresistance in sporadic breast cancer. By performing colony formation assays in response to Xray treatment we have shown that there is a wide range of sensitivities to this form of treatment. Interestingly, we have also observed that *p53* status does not accurately predict response to radiation treatment. Using correlation studies, we have identified novel predictors of IR resistance/sensitivity. We have sensitized two breast cancer cell lines to X-ray treatment by using RNAi-based technology to inhibit Artemis function. In the future, these observations may have a significant clinical impact on the way radiation oncologists treat breast cancer patients.

15. SUBJECT TERMS
Breast cancer, cell culture, radio-sensitivity/resistance, RNAi, DNA repair, X-ray treatment

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Research Objectives:

The overall goal of this research project is to develop an *in vitro* cell culture system to assess the relative radiation sensitivity in a panel of breast cancer cell lines in order to develop a high throughput system to identify novel targets that will further sensitize these cells to X-ray treatment.

Introduction:

It is estimated that over fifty percent of women each year diagnosed with breast cancer will receive radiation treatment. However, it is often not known if the tumors will respond to radio or chemotherapeutic treatment. Radio-therapy also has many side effects that directly affect quality of life such as skin scarring, hair/pigment loss, chronic pain and fatigue. In order to help treat breast cancer patients and give radiation-oncologists a more detailed model of the response of sporadic breast tumors to radiation treatment we proposed to develop an *in vitro* cell culture system that accurately mimics the response of sporadic breast tumors to radiation treatment.

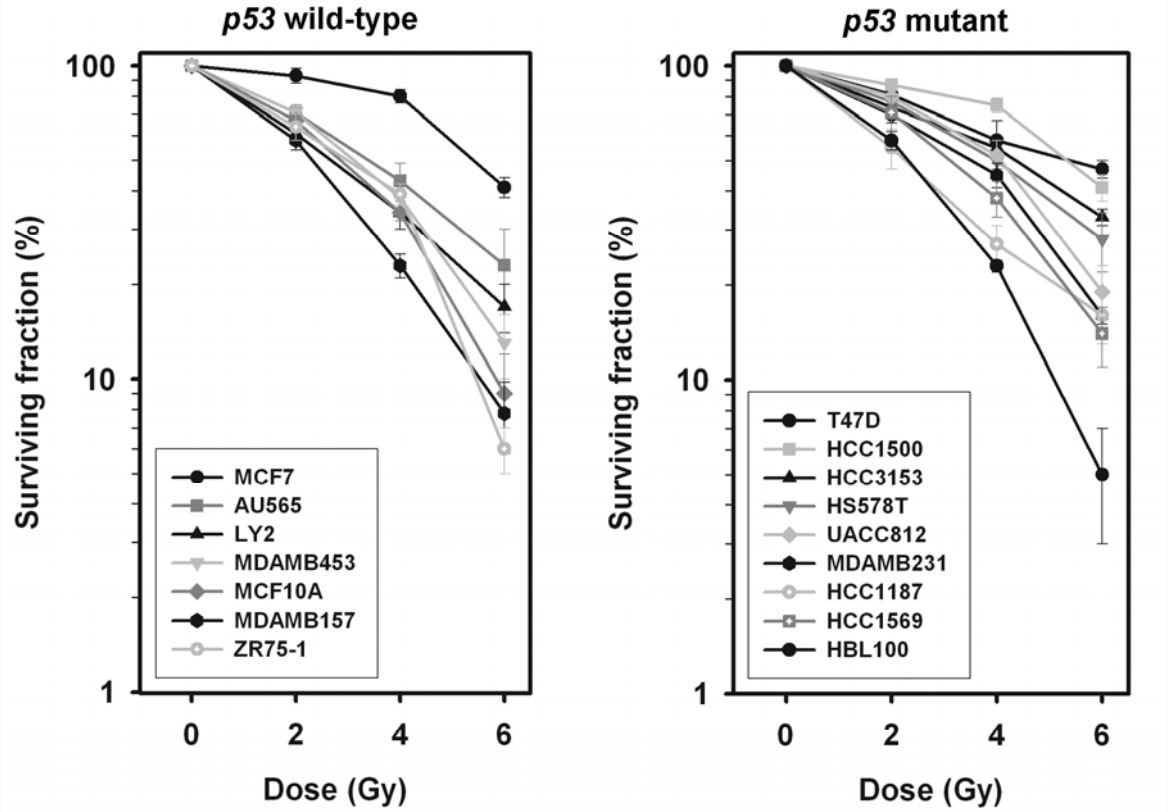

Figure 1: Breast cancer lines vary in their survival rates to IR exposure.
The breast cancer cell lines were divided into two groups based on *p53* status. These plots are based on clonogenic survival and are presented as the mean (+/- s.d.) values from three independent experiments.

In order to understand how breast tumors respond to radiation it is important to address how radiation works and how breast epithelial cells respond to IR treatment. IR is energy transmitted in the form of electrons that pass through the cell and cause damage to the DNA in the form of double strand breaks along with other forms of DNA damage. In a normal cell, there are stress response pathways and DNA repair pathways that are activated independent of each other. It is well known that *p53* is responsible for activating genes that stall the cell cycle to allow the DNA to be repaired before entering back into the cell cycle. While this cell is stalled in G_1 or G_0, the predominant DNA repair pathway activated is the non-homologous end-joining system which is directly responsible for the religation of the DNA double-strand breaks. This pathway is somewhat error-prone and a few nucleotides of sequence may be lost in this process. To add another layer of complexity to this field, the response of cells to X-rays is at least partially cell-type specific. Some

cell-types such as lymphoblasts are very sensitive to X-ray exposure and quickly undergo apoptosis. While epithelial cells do not just enter an apoptotic pathway in response to radiation, but are also known to senesce and undergo mitotic catastrophe. Due to the heterogeneity of sporadic breast cancer genomes many of the stress response and DNA repair pathways are either non-functional or aberrant and this would suggest that there will be a range of responses to X-ray treatment.

Results:

Aim 1: To determine the relative IR-sensitivity of 55 human breast tumor and DCIS-derived cell lines as well as normal HMEC cultures.

Measuring the relative radio-sensitivity of the breast cancer cell lines:

TABLE 1:

Cell line	percent survival at 6 Gy	gene cluster	p53 status	Tumor type
HBL100	5%	BaB	mutant	N
ZR75-1	6%	Lu	wild-type	IDC
MDAMB157	8%	BaB	wild-type	MC
MCF10A	9%	Lu	wild-type	F
MDAMB453	13%	Lu	wild-type	AC
HCC1569	14%	BaB	mutant	MC
HCC1187	16%	BaA	mutant	Duc,CA
MDAMB231	16%	BaB	mutant	AC
LY2	17%	Lu	wild-type	PE
UACC812	19%	Lu	mutant	IDC
AU565	23%	Lu	wild-type	AC
HS578T	28%	BaB	mutant	IDC
HCC3153	35%	BaA	mutant	not reported
HCC1500	41%	BaB	mutant	MC
MCF7	41%	Lu	wild-type	IDC
T47D	47%	Lu	mutant	IDC

Using traditional colony formation assays we have determined the relative sensitivity and resistance to s subset of our breast cancer cell line panel that will allow us to evaluate genes known to predict radio-responses in other cancer types such as *p53* status. More importantly, we can use statistical analyses to identify novel predictors of radio-resistance and sensitivity. Briefly each cell line was plated in either 200 or 600 cells per well in a six-well plate for low doses (0 or 2 Gy) or plated at 1000 or 5000 cells per well for higher doses of X-rays (4 and 6 Gy). The six-well plates were placed back into the incubator for ten to fourteen days for colonies to form. Once stained with crystal violet, the colonies were counted and plotted. These experiments were conducted at least two other times to generate true biological triplicates.

As presented in **Table 1**, there is almost a ten-fold difference in the most IR-sensitive breast cancer cell line (HBL100) and the most IR resistant cell line tested T47D. Our data also suggests that *p53* status is poorly correlated with response to X-ray treatment. Basal or luminal status of the cell line also does not seem to be an accurate predictor for IR response. Since the Gray laboratory has determined the relative transcript levels of roughly 22,000 genes by use of Affymetrix U133A chip analysis. We decided to perform simple correlation studies to determine which gene expression profiles are highly correlated

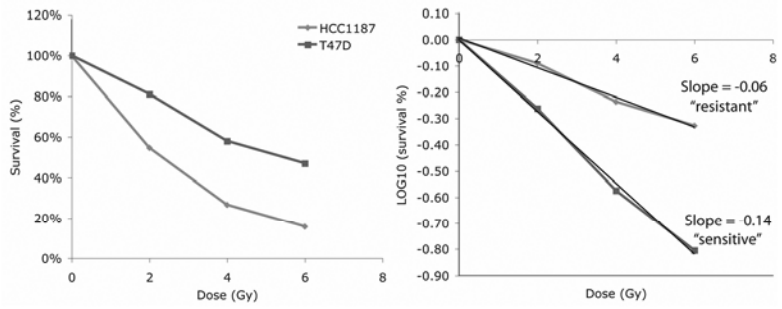

Figure 2: Abstracting the survival curves into a single continuous variable. The dosage in Gy remains constant and the y-axis is converted to log10 of the percent survival. The slope of this plot is then calculated and used as our continous variable for relative IR resistance or sensitivity.

with IR-resistance and sensitivity. Since response to IR is generally a quadratic-linear based-equation the first step in performing our correlation analysis was to convert our survival data into one continuous variable. We converted the curves into a continuous variable by keeping the dosage in Gy constant and the y-axis was converted to log 10 of the percent survival. The slope of this plot was then used as our continuous variable to represent survival. Next we setup our parameters for the Pearson's correlation between the slope generated from the survival data and the relative expression levels of the Affymetrix data. Our only cutoff was to exclude genes that had less than a two-fold change over our sub-panel of sixteen breast cancer cell lines.

TABLE 2

IR-resistance	Chromosomal location	Gene symbol	Subcellular localization	Function	Pearson's correlation
1	17	ATP6V0A1	CELLULAR	ION TRANSPORT	0.79
2	3	PLA1A	CELLULAR	LIPID METABOLISM	0.79
3	9	SYK	NUCLEAR	AKT PATHWAY	0.76
4	19	CLPTM1	CYTOPLASMIC	DEVELOPMENTAL	0.75
5	11	FLJ11848	UNKNOWN	UNKNOWN	0.75
6	15	PML	NUCLEAR	DNA REPAIR	0.74
7	1	ELOVL1	CYTOPLASMIC	LIPID METABOLISM	0.73
8	2	KIAA0436	UNKNOWN	UNKNOWN	0.72
9	11	DPAGT1	CYTOPLASMIC	POST-TRANSLATIONAL MODIFICATION	0.72
10	17	ABCA5	CYTOPLASMIC	MULTIDRUG RESISTANCE	0.71
IR-sensitivity	**Chromosomal location**	**Gene symbol**	**Subcellular localization**	**Function**	**Pearson's correlation**
1	2	PPIG	NUCLEAR	RNA SPLICING	-0.77
2	2	FACL3	CYTOPLASMIC	LIPID METABOLISM	-0.77
3	19	NDUFA7	CYTOPLASMIC	MITOCHONDRIAL	-0.76
4		NA	UNKNOWN	UNKNOWN	-0.75
5	16	RBBP6	NUCLEAR	E3 LIGASE	-0.74
6	1	KIAA0485	UNKNOWN	UNKNOWN	-0.73
7	5	RY1	NUCLEAR	UNKNOWN	-0.73
8	21	DYRK1A	NUCLEAR	UNKNOWN	-0.72
9	11	CDKN1C	NUCLEAR	CELL CYCLE	-0.72
10	18	RAB27B	CYTOPLASMIC	VESICLE TRAFFICKING	-0.72

Table 2 depicts the top ten genes whose transcript levels positively correlated with radio-resistance and our top ten genes whose transcript levels negatively correlated with IR-resistance therefore we categorized these genes as being IR-sensitivity genes. Interestingly, in our top ten genes associated with radio-resistance some are genes known to be associated with radio-resistance and others are novel. For example, CLPTM1 is a gene when mutated causes cleft palate but as reported by Folgueira et al with two other genes was able to correctly classify greater than 95% of clinical breast tumor samples in responsiveness to doxorubicin treatment (Folgueira, Carraro et al. 2005). Our number one gene for IR resistance was ATP6VOA1 which is a vacuolar H(+)ATPase.

This is quite interesting since it is well documented in the literature that ouabain inhibition of sodium, potassium pumps sensitizes tumor cells to IR treatment (Lawrence 1988; Verheye-Dua and Bohm 1996; Verheye-Dua and Bohm 1998). Several lipid metabolism genes appeared on our list (PLA1A, ELOVL1 and FACL3) which further supports previous studies that demonstrated inhibition of fatty acid synthesis suppresses HER2 overexpression and increases apoptotic rates (Menendez, Lupu et al. 2004; Menendez, Vellon et al. 2004; Menendez and Lupu 2005) and also suggests a novel function for fatty acid metabolism. ELOVL1 is essential for long chain fatty acid synthesis and is associated with IR resistance, while FACL3 is known to inhibit fatty acid synthesis and ligate acyl-CoAs to fatty acids (Qiao and Tuohimaa 2004) and is associated with IR sensitivity. Many of these candidates warrant more detailed investigation, which is currently being pursued by direct transcript measurement by Panomics Inc. and by siRNA-directed inhibition of these radio-resistance associated genes.

Western Blot Analysis of NHEJ Members- Potential Predictors of IR-Sensitivity.

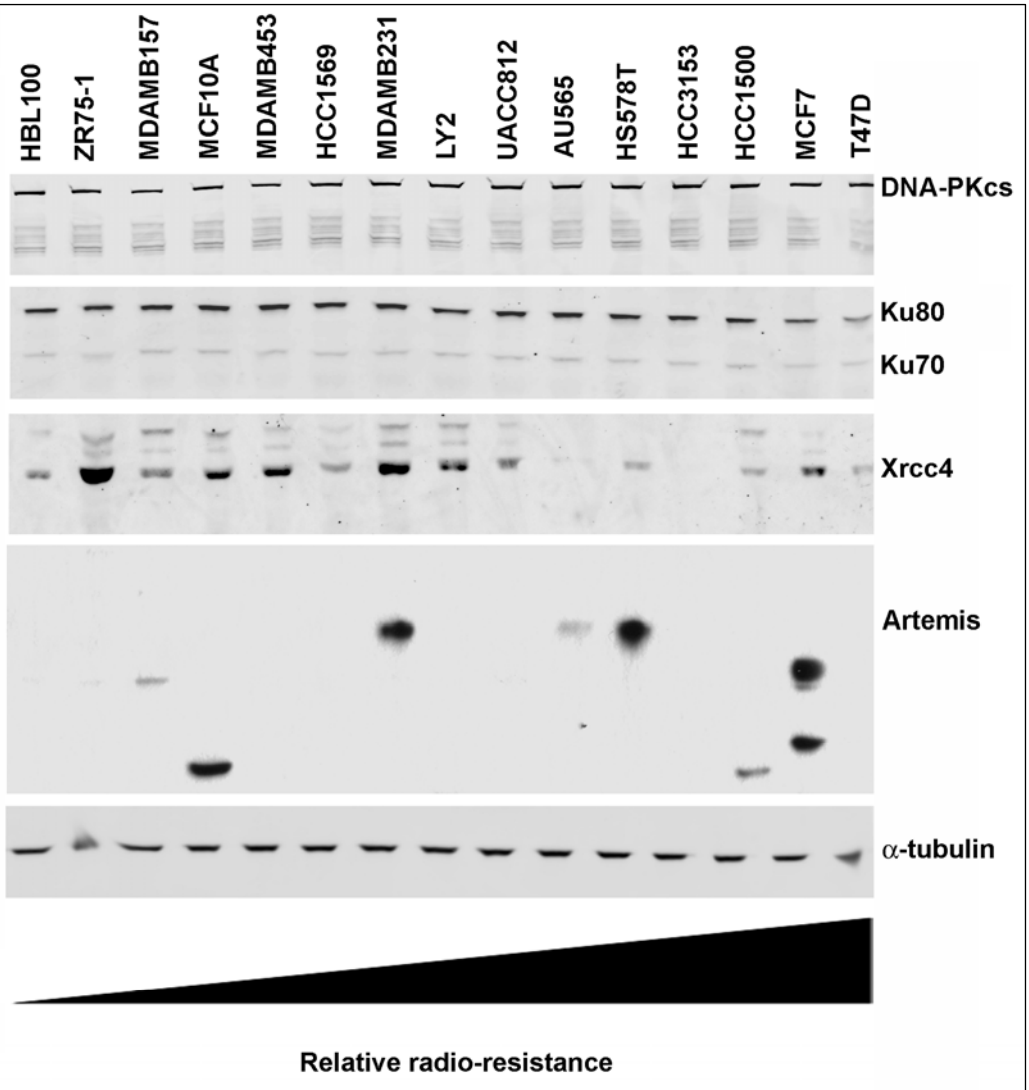

Figure 3: Variation of protein expression patterns of XRCC4 and Artemis in Breast Cancer Cell LInes. 50 *u*g of total protein lysate was resolved byBis-Tris SDS PAGE, transfered to nitrocellulose membrane and probed with antibodies specific for the NHEJ proteins depicted above.

Since the NHEJ pathway is essential to repair radiation-induced double-strand breaks, we decided to interrogate the relative protein levels of key components of this pathway. As depicted in **Figure 3**, there was little variation in DNA-PK holoenzyme levels which includes DNA-PKcs and the Ku70/80 heterodimer.

However, we did observe significant differences in both XRCC4 and Artemis protein levels. Merel et. al. observed differences in XRCC4 in breast cancer cell lines, and we are the first to observe differences in the levels of expression of the Artemis protein (Merel, Prieur et al. 2002). There were also differences in the migration pattern of Artemis strongly suggesting that different splice variants are

expressed in the mammary epithelium or we maybe detecting degradation products. Unfortunately, there was no strong correlation between radio-sensitivity/resistance and the relative protein levels of XRCC4 or Artemis.

Aim 2: To utilize RNAi-based methodologies in order to specifically knock-down key NHEJ genes (Artemis or DNA-PK) to increase IR sensitivity of selected breast cancer cell lines.

RNAi of Artemis in HBL100 and MDAMB231 cell lines increases IR sensitivity

In Aim 2 of this project we have made significant progress by creating stable cell lines from HBL100 and MDAMB231 that express shRNAs targeting the Artemis transcript. We have also generated stable cell lines that expressing a scrambled shRNA directed against AP2. Once these stable lines were generated, we performed semi-quantitative RT-PCR to determine the efficiency of the Artemis transcript knock-downs. As depicted in **Figure 4**, shRNA #1 is more effective at knocking-down Artemis levels than shRNA #2. shRNA-scrambled AP2 had no significant effect on relative Artemis transcript levels. Western blot analysis confirmed our RT-PCR observations depicting that our shRNA constructs are effective at knocking down Artemis expression levels. Survival assays confirm that the reduction of Artemis expression levels increases radiation sensitivity in the two breast cancer cell lines tested.

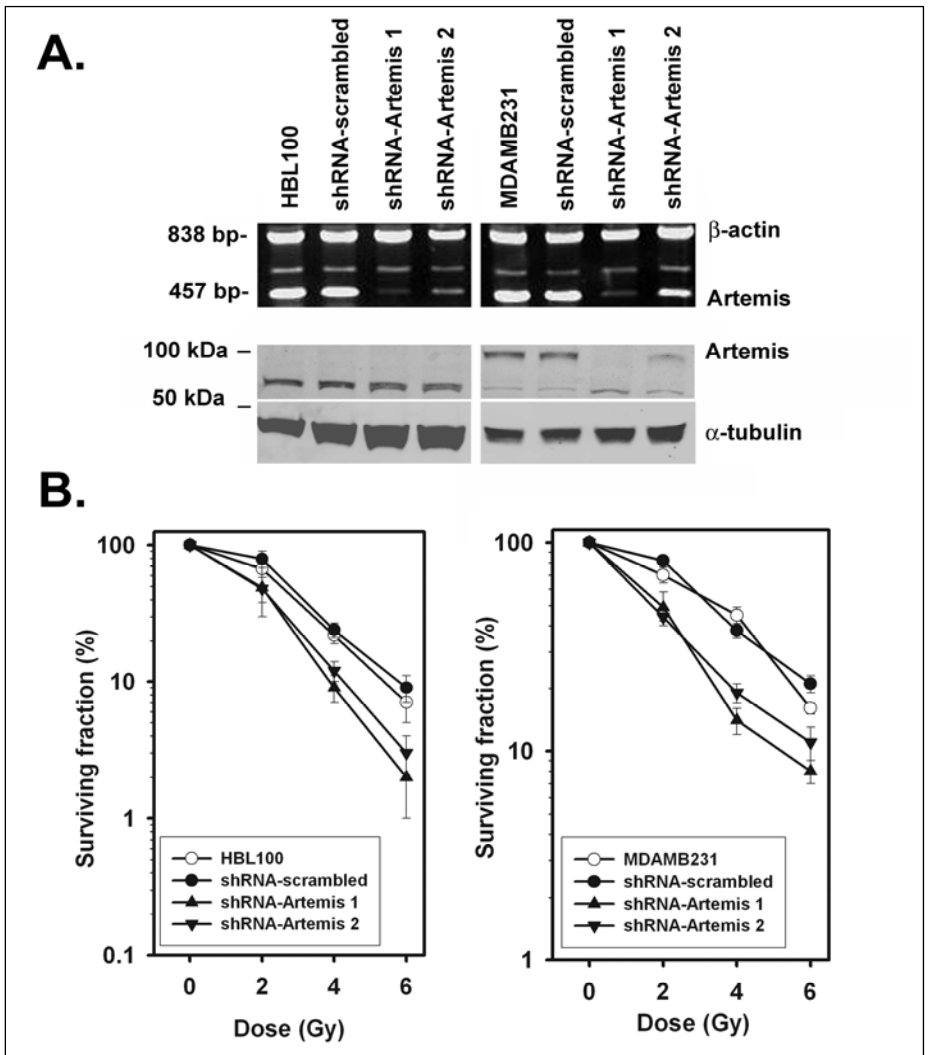

Figure 4: RNAi of Artemis Expression in Breast Cancer Cell Lines Increases IR Sensitivity. **A.** Semi quantitative RT-PCR experiments show a reduction in Artemis transcript levels in cell lines expressing shRNAs targeting the Artemis transcript as compared to the parental and scrambled hairpin control. β-actin was used as a loading control. Western blot analysis shows the relative Artemis protein levels in the HBL100 and MDAMB231 cell lines. Both shRNA-Artemis 1 and 2 reduce Artemis protein levels. **B.** Clonogenic survival assays of HBL100 and MDAMB231 parental cell lines and those that constitutively express shRNAs targeting Artemis or a scrambled hairpin targeting AP2 (negative control). Asynchronous cultures were seeded in triplicate, irradiated and incubated for 10-14 days, and the resulting colonies were stained and scored.

Key Research Accomplishments:

We have established survival curves to sixteen breast cancer cell lines in response to IR treatment. *p53* status, luminal or basal cell subtype, NHEJ protein levels are all poor predictors of IR sensitivity. Using correlative analysis, we have identified a novel gene set of predictors of IR resistance/sensitivity which will be of interest to radiation oncologists and radiation biologists. We have established RNAi of Artemis increases IR sensitivity in two breast cancer cell lines MDAMB231 and HBL100.

Reportable Outcomes:

By utilizing colony formation assays we have detected up to ten-fold differences of breast cancer cell lines by measuring survival after IR exposure. These differences do not correlate with cell type classification, *p53* status, NHEJ protein levels or overall growth rate. We have identified a gene set that strongly correlates with IR-resistance/sensitivity. Many of these genes have not been previously implicated in radio-resistance/ sensitivity. Finally, two different breast cancer cell lines have been sensitized to IR treatment by knock-down of the Artemis protein.

Future Directions:

In order to test our *in vitro* observations, we are going to an *in vivo* system. By using mouse xenograph studies, we determine if Artemis targeted breast cancer cell lines by both shRNA and siRNAs are more sensitive to IR treatment.

Aim 3: Validation of our *in vitro* cell-based system to assay radiation sensitivity in breast cancer by using mouse xenograph models.

Summary of Aim:

We will use select breast cancer cell lines that readily form tumors in nude mice (MDAMB231 and MCF7) to test the use of lentiviral shRNA constructs and therapeutic DNA repair gene targeting using neutral liposomal small interfering RNA Delivery. These tumors will be irradiated to determine if the inhibition of specific DNA repair genes reduce tumor size *in vivo* after X-ray treatment.

Specific Aim 3:

Xenograph studies of breast cancer cell lines depleted of Artemis and/or DNA-PK by constitutive shRNA expression or by therapeutic DNA repair gene targeting *in vivo* using neutral liposomal small interfering RNA delivery.

Experiment #1:

In the first experiment, we will determine if the parental MDAMB231 breast cancer cell line is more radio-resistant than established cell lines MDAMB231-4 and MDAMB231-5 (shRNA directed towards Artemis) or MDAMB231-shRNA-scrambled AP2 and there relative sensitivities to X-ray exposure.

Statistical considerations:

Since we are hypothesizing a 50% reduction in tumor size as observed *in vitro* (β error=0.2), ten mice for each group will be used as directed by a power analysis. Continuous variables such as tumor size will be analyzed for statistical significance (achieved if $P < 0.05$) with the Student's t test for two group comparisons and ANOVA will be used for multi-group comparisons.

Induction of breast tumors:

Female athymic nude mice (NCr-*nu*) will be purchased from the National Cancer Institute-Frederick Cancer Center. Each of the different cell lines mentioned in the previous paragraph will be

cultured in 10% FBS DMEM. Cells will be harvested by trypsinization and washed with sterile PBS and resuspended to a concentration of 1 x 10^8 cells/mL. The cells will be injected subcutaneously into the right thigh area of the nude mice. Tumor growth will be monitored by caliper measurements (palpation).

X-ray treatment of xenograph tumors and tumor analysis:

Once tumor diameter reaches 0.5cm^3, the mice will be anesthetized and provided with lead-based shielding to protect internal organs such as heart, lung, liver and intestines from unwanted radiation exposure. The tumor will be exposed to one acute dose of medium-level X-rays (60kV/10mA) at 2 Gy or 4 Gy and tumor growth will be continued for 15 to 30 more days. These mice will be euthanized and tumors removed and weighed. Student's t test will be used to determine if there is a significant difference in tumor mass between parental and shRNA expressing cell lines.

Experiment #2:

The goal of this experiment will be to utilize siRNAs as radio-therapeutic in our cell culture/xenograph model.

Previous studies demonstrate that ovarian cancer can be reduced by siRNA directed towards the *EphA2* gene combined with paclitaxel treatment (Landen, Chavez-Reyes et al. 2005). These siRNAs were coated with a neutral liposome technology that has proven to improve *in vivo* targeting efficiency. In pilot experiments, we will also directly inject naked siRNAs and naked liposomes as a negative control.

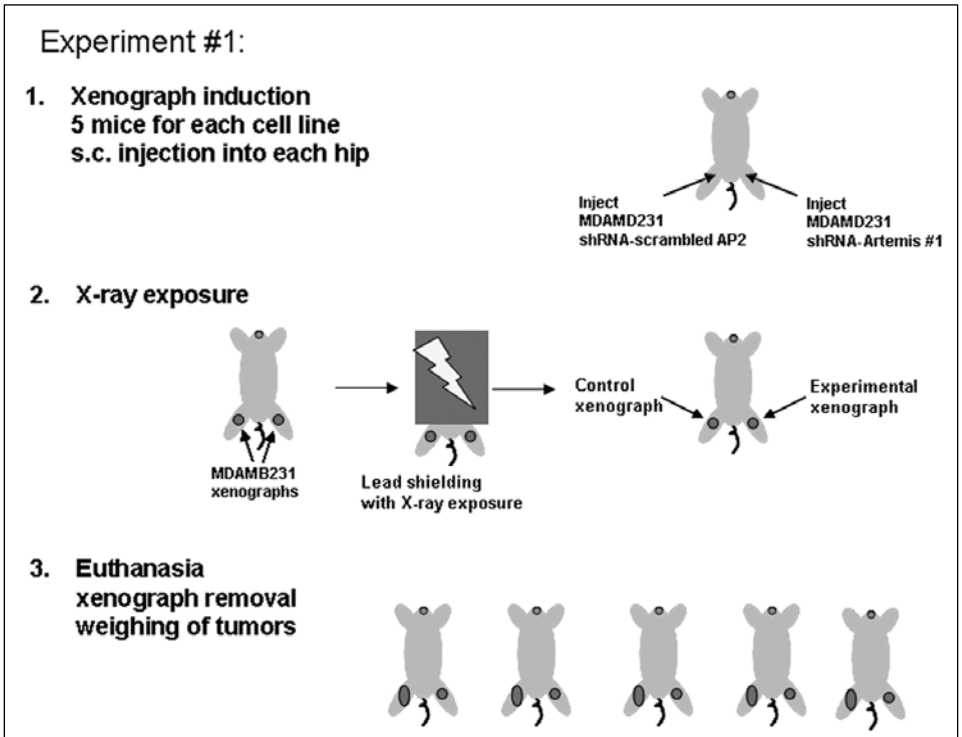

Figure 5: Outline of first mouse xenograph experiment
In the first experiment, we will determine if the parental MDAMB231 breast cancer cell line is more IR-resistant than established cell lines MDAMB231-shRNA-1 and 2 (targeting Artemis) or MDAMB231-shRNA-scambled AP2. Briefly, we will generate xenogrpahs of the different cell lines, once tumors are formed, we will irradiate each mouse, and allow tumors to grow for two to four more weeks. We will then move on to step three and euthanize the mice, remove and weigh each tumor. Statistical analysis will then be performed.

Experiment #2:

In the second experiment, we will determine if we can reduce tumor size of breast cancer xenographs using DNA repair gene targeting *in vivo* using liposomal small interfering RNA delivery. Specifically, we will generate xenographs as described in Experiment #1 of the parental cell lines MDAMB231 and MCF-7.

Liposomal/siRNA preparation, injection and X-ray exposure:

siRNAs tagged with Alexa 555 (Qiagen) will be coated in the neutral liposome 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) by Anil Sood's laboratory at the Texas M.D. Anderson Medical Center, Houston, TX. We will inject these neutral coated liposomes with siRNAs, naked siRNAs (150ug/kg) or empty liposomes directly into the tumor site. Three days after injection, the tumors will be dosed with X-rays as directed by results from Experiment #1 (4 Gy). Mice (*n*=10 per group) will be monitored for adverse effects and tumors will be harvested 3-4 weeks after X-ray treatment. Mouse weight, tumor weight and distribution of the tumor will be recorded. Vital organs will be harvested and necropsies will be performed by a board-certified veterinarian for evidence of tissue toxicity.

Conclusions:

Our *in vitro* results suggest that RNAi targeting of Artemis will sensitize the xenograph tumors to IR treatment. These results would confirm our previous *in vitro* results and provide more evidence that our *in vitro* cell-culture based system to assay radiation sensitivity in breast cancer. We could then perform more rapid screens of other siRNAs or small molecule inhibitors specifically designed to the gene list described in **Table 2** to further sensitize breast tumors to radio- and chemotherapeutic treatments. Additionally, siRNA therapy could also be used to shrink the tumor before debulking therapy and if the tumors are small enough it might be possible in the near future to treat with siRNAs, radio and chemo-therapeutic therapies and eliminate the breast tumor without having to perform invasive surgery or mastectomies.

Materials and Methods:

Cell Culture:

All breast cancer cell lines were kindly provided by the Gray Laboratory or purchased directly from ATCC. Fetal bovine serum, basal medium, trypsin-EDTA and phosphate buffered saline were purchased from JRS Scientific. The cells were cultured at 37° C, 90% humidity supplemented with 5% CO_2.

Colony survival assays:

Cells were de-attached from their dishes by trypsin incubation and counted using a Coulter counter. For each X-ray dose, cells were plated at both 1000 and 5000 cells per well in triplicate. The cells were allowed to attach overnight and were exposed to X-rays using a Pantek X-ray machine at 320kV/10mA. The doses given were 0, 2, 4 and 6 Gy. The cells were placed in the incubator and incubated for ten to fifteen days to allow colonies to form. The colonies were then examined using phase-contrast microscopy to determine if there were approximately fifty cells per colony. The colonies were fixed and stained with a crystal violet solution and counted by hand. The percent survival was calculated using Microsoft Excel and curves subsequently plotted in semi-log scale

Lentiviral small hairpin RNA (shRNA) construct generation:

In order to efficiently knockdown Artemis expression shRNAs were designed according to standard algorithms. The oligos were synthezied by Operon technologies, allowed to anneal and cloned into a modified pENTRY vector. The pENTRY vectors were sequenced to verify the sequence of the shRNAs and propagated in the *E. coli* strain STBL3. LR reactions were performed according to the manufacturer's protocol (Invitrogen) and this allows the transfer of the shRNA into the Lenti-destination vector. These vectors were transfected into HEK293FT cells along with the helper plasmids necessary for lentiviral production. Lentiviral particles were purified using standard centrifugation techniques and stored at -80° C.

Selection ofHBL100 and MDAMB231 cells expressing the shRNAs directed against Artemis:

MDAMB231 cells were infected with lentivirus for forty eight hours and then placed under antibiotic selection for two weeks. The stable clones are then analyzed for Artemis expression or lack there of by western blot analysis. Survival assays were performed on these cell lines according to the methods stated previously.

RNA Preparation and RT-PCR analysis of Artemis and β-actin expression:

Total RNA from cultured cells was isolated using TRIzol LS Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad,CA). For reverse transcription-polymerase chain reaction (RT-PCR), Im-Prom-II™ Reverse Transcriptase was used according to the supplier's instructions (Promega, Madison,WI). First-strand cDNA was synthesized from 5*u*g of total RNA using both oligodT and random primers and was carried out at 50°C for thirty minutes in 20*u*L reaction. 2*u*L of cDNA was the starting material for Artemis specific primers and β-actin control primers (Bio-chain Institute, Inc., Hayward,CA). PCR reactions were performed according to the manufacturer's PCR protocol (Bio-chain Institute, Inc., Hayward,CA). Briefly, the PCR reaction consisted of a five minute denaturation, followed by 30 cycles of 94°C x 30 sec., 56°C x 30 seconds, 72°C x 30 sec. and a final extension at 72°C for seven minutes. The PCR products were visualized by resolving on a 1% (0.5% TBE) agarose gel with ethidium bromide and visualized under UV light.

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